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(57) Abstract

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METHODS FOR DETECTING CANCER-ASSOCIATED CHROMOSOME DELETIONS USING IN SITU HYBRIDIZATION

BACKGROUND OF THE INVENTION

The present invention provides accurate and efficient means of detecting chromosomal deletions associated with neoplastic growth. In particular, it provides methods and composition for the localization of precise regions of loss of heterozygosity on chromosome 16q in breast tumor cells, and identifying putative tumor suppressor gene(s) encoded by this region.

Genetic aberrations in a single cell are currently hypothesized to give rise to cancer (Nowell, *Science* 194:23 (1976), Foulds, *J. Chronic Dis.* 8:2(1958). Identification of the genetic events leading to neoplastic transformation and subsequent progression can facilitate efforts to define the biological basis for disease, improve prognostication and prediction of therapeutic response, and permit earlier tumor detection.

In particular, the deletion or multiplication of copies of whole chromosomes or chromosomal segments, and higher level amplifications of specific regions of the genome are common occurrences in cancer. See, for example, Smith, *et al.*, *Breast Cancer Res. Treat.*, 18: Suppl. 1: 5-14 (1991), van de Vijer & Nusse, *Biochim. Biophys. Acta.* 1072: 33-50 (1991), Sato, *et al.*, *Cancer. Res.*, 50: 7184-7189 (1990). In fact, the amplification and deletion of DNA sequences containing proto-oncogenes and tumor-suppressor genes, respectively, are frequently characteristic of tumorigenesis. Dutrillaux, *et al.*, *Cancer Genet. Cytogenet.*, 49: 203-217 (1990). Clearly the identification of amplified and deleted regions and the cloning of the genes involved is crucial both to the study of tumorigenesis and to the development of cancer diagnostics.

One model of carcinogenesis involves the inactivation of tumor suppressor genes that, in noncancerous cells, are believed to regulate cellular proliferation, differentiation or morphogenesis. Inactivation of these genes can lead to uncontrolled cell proliferation, resulting in carcinogenesis. (Hollingsworth *et al.*, *J. Natl Cancer Inst.* 83, 91-96 (1991); Solomon *et al.*, *Science* 254, 1153-1160 (1991)). Typically, carcinogenesis requires that both alleles of a tumor suppressor gene be inactivated (Knudson, *Proc. Natl. Acad. Sci. USA* 68, 820-823 (1971)). Often, inactivation occurs by mutation of one allele and loss of the other as a consequence of either mitotic recombination or physical deletion. Consequently, tumors carrying mutant tumor suppressor genes often show loss of heterozygosity (LOH) near the site of the suppressor gene. Several tumor suppressor genes, for example, RB1 (13q14.2), TP53 (17p13.1), WT1 (11p13), APC (5q21-22), and DCC (18q21.1), have been identified in regions showing LOH.

In breast cancer, loss of heterozygosity has been reported on chromosomes 1p, 1q, 3p, 6q, 8q, 9q, 11p, 13q, 15q, 16q, 17p, 17q, 18p, and 22q (Devilee *et al.*, *Oncogene* 6: 1705-1711 (1991), Sato *et al.*, *Cancer Res.* 51: 5794-5799 (1991), Larsson *et al.*, *Genes, Chromosomes and Cancer* 2: 191-197 (1990)). LOH on chromosome 16q is of particular interest for several reasons. First, three different groups have reported a high incidence of LOH on chromosome 16q in cells derived from breast cancer tumors: 40% (Devilee *et al.*, *supra.*), 51% (Sato *et al.*, *supra.*), and 53% (Larsson *et al.*, *supra.*). Second, LOH occurs on chromosome 16q in other tumors, including hepatocellular carcinoma, prostate cancer, and Wilms' tumor (Tsuda *et al.*, *Proc. Natl. Acad. Sci. USA* 87: 691-694 (1990), Nishida *et al.*, *Int. J.Cancer* 51: 862-868 (1992), Carter *et al.*, *Proc. Natl. Acad. Sci. USA*

87: 8751-8755 (1990), Bergerheim *et al.*, *Genes, Chromosomes and Cancer* 3: 215-220 (1991), Maw *et al.*, *Cancer Res.* 52: 3094-3098 (1992)). Moreover, reduced relative gene copy number has been detected on chromosome 16q in breast cancer, prostate cancer and ovarian cancer using comparative genomic hybridization (CGH) (Kallioneimi *et al*, *Science* 258: 818-821 (1992)).

The particular genetic aberrations found in a particular breast tumor can have a profound effect on the prognosis and optimal treatment. The life expectancy of breast cancer patients varies from a relatively short time to as much as forty years (Rutqvist *et al.*, *Cancer* 55: 658-665 (1985)), and is at least partially dependent upon the particular genetic changes that give rise to the tumor. For example, LOH at the MET locus on chromosome 7q is associated with aggressive disease in one subset of breast tumors (Bieche *et al.*, *Lancet* 339: 139-143 (1992)). Metastases from breast to the lymph nodes is increased when the NM23 tumor suppressor gene is lost from chromosome 17q (Hennessy *et al.*, *J. Int. Nat'l Cancer Inst.* 83: 281-285 (1991). Therefore, diagnosis and treatment of breast cancer depend upon the availability of accurate and efficient methods for determining the genetic aberrations that give rise to the particular cancer.

Cytogenetics is the traditional method for detecting amplified or deleted chromosomal regions. The resolution of cytogenetic techniques is limited, however, to regions larger than approximately 10 Mb (approximately the width of a band in Giemsa-stained chromosomes) because of the complex packing of DNA into the chromosomes. In complex karyotypes with multiple translocations and other genetic changes, traditional cytogenetic analysis is of little utility because karyotype information is lacking or cannot be interpreted. Teyssier, J.R., *Cancer Genet. Cytogenet.*,

37: 103 (1989). Furthermore conventional cytogenetic banding analysis is time consuming, labor intensive, and frequently difficult or impossible.

More recent methods for assessing the amount of a given DNA sequence in a chromosome are more sensitive than the traditional cytogenetic techniques. These methods employ cloned DNA or RNA probes that are hybridized to chromosomal DNA. This method is effective even if the genome is heavily rearranged so as to eliminate useful karyotype information. However, this Southern blotting method gives only a rough estimate of the copy number of a DNA sequence, and does not provide any information about the localization of that sequence within the chromosome.

Comparative genomic hybridization (CGH) is a more recent approach to identify the presence and localization of amplified or deleted sequences. *See*, Kallioniemi *et al.*, *supra*. CGH, like Southern blotting, reveals amplifications and deletions irrespective of genome rearrangement. CGH has advantages over Southern blotting, providing a more quantitative estimate of copy number, and also providing the location of the amplified or deleted sequence in the normal chromosome. Where a deletion or amplification is limited to the loss or gain of one copy of a sequence, the CGH resolution is usually about 5-10 Mb. Furthermore, the sensitivity of both CGH and Southern blotting techniques to contamination of tumor samples by normal cells makes estimating the copy number of particular chromosomal sequences within the tumor cell population very difficult.

New techniques which provide increased sensitivity, more precise localization of a deletion associated with LOH and neoplastic growth are particularly desirable for the diagnosis of cancer. The present invention provides these and other benefits.

SUMMARY OF THE INVENTION

The present invention provides methods and compositions for detecting deletions associated with neoplastic growth. The methods comprise contacting a chromosome sample from a patient with a composition consisting essentially of one or more probes each of which binds selectively to a target polynucleotide sequence on human chromosome 16 distal to 16q21 under conditions in which the probe forms a stable hybridization complex with the sequence. The hybridization complex is then detected using standard techniques, typically by FISH.

The probe is usually labeled with digoxigenin or biotin, which is visualized by detecting a fluorescent label, such as FITC. In preferred embodiments, the probe comprises a polynucleotide sequence from C52, C196 and C21.

The hybridization complex is usually detected in a metaphase cell in a sample from a breast tumor. In addition, reference specific to chromosome 16 centromere may be used.

To confirm a loss of heterozygosity, the methods may also include detecting a mutant allele of a gene in the deleted region of chromosome 16. For instance, E-cadherin expression can be determined. Other methods for detecting a mutant allele include PCR.

Also provided are nucleic acid probes which bind selectively to a polynucleotide sequence on human chromosome 16 distal to 16q21. The probes are typically labeled with digoxigenin or biotin. Preferred probes comprise polynucleotide sequences from C52, C196 and C21.

The invention further provides kits comprising a compartment which contains a nucleic acid probe which binds substantially a polynucleotide on human chromosome 16 distal to 16q21. The probes are

preferably labeled as described above. The kits may also comprise compounds for the detection of the probes, such as Texas red Avidin and biotin-labeled goat anti-avidin antibodies.

Definitions

A "chromosome sample" as used herein refers to a tissue or cell sample prepared for standard *in situ* hybridization methods described below. The sample is prepared such that individual chromosomes remain substantially intact and typically comprises metaphase spreads or interphase nuclei prepared according to standard techniques. The tissue sample from which the chromosome sample is prepared is typically taken from a patient suspected of having the disease associated with the deletion being detected.

As used herein a "probe" is defined as a polynucleotide (either RNA or DNA) capable of binding to a complementary target cellular genetic sequence through one or more types of chemical bonds, usually through hydrogen bond formation. It will be understood by one of skill in the art that probes will typically substantially bind target sequences lacking complete complementarity with the probe sequence depending upon the stringency of the hybridization conditions. The probes are preferably directly labelled as with isotopes or indirectly labelled such as with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the target. Nucleic acid probes can be prepared by a variety of methods known to those of skill in the art.

A "composition consisting essentially of one or more probes each of which binds selectively to a target polynucleotide sequence" refers to a collection of one or more probes which bind substantially to the target sequence and nowhere else in the target

chromosome or genome and which allow the detection of the presence or absence of the target sequence. Such a composition may contain other nucleic acids which do not materially affect the detection of the target sequence. Such additional nucleic acids include reference probes specific to a sequence in the centromere in the chromosome.

"Bind(s) substantially" refers to complementary hybridization between an oligonucleotide and a target sequence and embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired detection of the target polynucleotide sequence.

"Hybridizing" refers the binding of two single stranded nucleic acids via complementary base pairing.

"Nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, would encompass known analogs of natural nucleotides that can function in a similar manner as naturally occurring nucleotides.

One of skill will recognize that the precise sequence of the particular probes described herein can be modified to a certain degree to produce probes that are "substantially identical" to the disclosed probes, but retain the ability to bind substantially to the target sequences. Such modifications are specifically covered by reference to the individual probes herein. The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 90% sequence identity, and more preferably at least 95%, compared to a reference sequence using the methods described below using standard parameters.

Two nucleic acid sequences are said to be "identical" if the sequence of nucleotides in the two

sequences is the same when aligned for maximum correspondence as described below. The term "complementary to" is used herein to mean that the complementary sequence is identical to all or a portion of a reference polynucleotide sequence.

Sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two sequences over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a segment of at least about 20 contiguous positions, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson and Lipman *Proc. Natl. Acad. Sci. (U.S.A.)* 85: 2444 (1988), by computerized implementations of these algorithms.

"Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of

comparison and multiplying the result by 100 to yield the percentage of sequence identity.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to the same sequence under stringent conditions. Stringent conditions are sequence dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 5° C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is at least about 0.02 molar at pH 7 and the temperature is at least about 60°C.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Shows the results of an experiment examining intratumoral heterogeneity in tumor B208.

Figure 2. Results of deletion mapping on chromosome 16q in 23 primary breast cancer samples. Closed boxes indicate deleted loci and open boxes indicate retained loci. Deletions were detected by fluorescence *in situ* hybridization.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention is based on the use of libraries of genomic probes form chromosome 16 in *in situ* hybridization to chromosome samples comprising interphase nuclei or metaphase spreads to detect chromosomal deletions. Generally, the method consists of two steps: 1) the creation of a mapped library of probes from chromosome 16, and 2) the *in situ* hybridization of those probes to a sample and subsequent detection of hybridization frequency to determine relative copy number of a particular

chromosomal region (typically those distal to 16q21) in the sample. The methods are preferably carried out using fluorophores to detect the presence of the hybridization complexes. These techniques are generally referred to as fluorescent *in situ* hybridization or FISH.

The mapped libraries of probes consist of a set of probes which when hybridized to a normal chromosome are distributed relatively uniformly across the region of interest. The region typically is one chromosome or a part of one chromosome.

FISH provides high resolution mapping of cancer-associated amplifications and deletions. As the physical mapping involved in the human genome project proceeds, more chromosomal probes are becoming available. In addition, FISH can be applied to archived material (Schofield *et al.*, *American Journal of Pathology* 141, 1265-1269 (1992)) or to the extremely small amounts of material available from fine needle aspirates or in touch preparations. Therefore, FISH adds substantially to the battery of techniques that can be used in locating genes involved in cancer progression.

Production of a Probe Library

To prepare a mapped library, a selected chromosome (*e.g.*, chromosome 16) is isolated by flow cytometry using methods well known to those of skill in the art. Briefly, chromosomes are isolated from cells blocked in metaphase by the addition *e.g.*, colcemid and stained with two DNA-binding fluorescent dyes. The stained chromosomes are then passed through a cell sorter and isolated using bivariate analysis of the chromosomes by size and base pair composition (*see, e.g.*, Blennow *et al.*, *Hum. Genet.* 90:371-374 (1992). The chromosome is then digested with restriction enzymes appropriate to give DNA sequences of at least about 20 kb and more preferably about 40 kb.

Techniques of partial sequence digestion are well known in the art. See, for example Perbal, *A Practical Guide to Molecular Cloning* 2nd Ed., Wiley N.Y. (1988). The resulting sequences are ligated with a vector which contains a resistance marker. The vector is transfected into and propagated in the appropriate host. Exemplary vectors suitable for this purpose include cosmids, yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs) and P1 phage. Typically, cosmid libraries are prepared. The cosmid library then consists of single clones of the transfected bacteria.

While it is possible to generate cosmid libraries, as described above, libraries spanning entire chromosomes are available commercially (Clonetech, South San Francisco, CA) or from the Los Alamos National Laboratory.

Mapping of Probe Library

Once a probe library is constructed, a subset of the probes is physically mapped on the selected chromosome, in this case chromosome 16. A number of techniques can be used to obtain a mapped library.

For instance overlapping sequences in a large number of randomly selected cosmid clones can be identified by unique restriction enzyme "fingerprinting" and their assembly into overlapping sets of clones. Such techniques have been used to map various nonhuman genomes (*see, e.g.*, Olson *et al.*, *Proc. Natl. Acad. Sci. USA* 83:7826 (1986) and Coulson *et al.*, *Proc. Natl. Acad. Sci. USA* 83:7821 (1986).

In addition, identification of overlapping clones by hybridization with RNA probes can be used. Cloning vectors are now available in which the promoters for a single polymerase, or for two different polymerases, lie adjacent to a cloning site. Transcription with any of the available polymerases enables one to produce large quantities of high-

specific activity RNA probes which correspond to either the coding or the non-coding strands (*see, e.g.* Wahl *et al.*, *Methods in Enzymology* 152:572 (1987)). See also U.S. Patent No. 5,219,726.

Alternately, FISH and digital image analysis can be used to localize cosmids along the desired chromosome. This method is described, for instance, in Lichter *et al.*, *Science*, 247:64-69 (1990). Briefly, the clones are mapped by FISH to metaphase spreads from normal cells using *e.g.*, FITC as the fluorophore. The chromosomes are counterstained by a stain which stains DNA irrespective of base composition (*e.g.*, propidium iodide or DAPI), to define the outlining of the chromosome. The stained metaphases are imaged in a fluorescence microscope with a polychromatic beam-splitter to avoid color-dependent image shifts. The different color images are acquired with a CCD camera and the digitized images are stored in a computer. A computer program is then used to calculate the chromosome axis, project the two (for single copy sequences) FITC signals perpendicularly onto this axis, and calculate the average fractional length from a defined position, typically the p-telomere. When the p-telomere is used as the reference point the position of each clone is expressed in units called FLpter.

For use in the present invention, a mapped library of probes for chromosome 16 is described by Stallings *et al.*, *Genomics* 13: 1031-1039 (1992).

Probe Preparation

The cosmid probes must be labeled for use in *in situ* hybridization. The probes may be detectably labeled prior to the hybridization reaction. Alternatively, a directly detectable label which binds to the hybridization product may be used. Such detectable group can be any material having a detectable physical or chemical property and have been well-developed in the field of immunoassays. Thus a

label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. Useful labels in the present invention include 32P, fluorescent dyes, electron-dense reagents, enzymes (as commonly used in an ELISA). Examples of labels which are not directly detected but are detected through the use of directly detectable label include biotin and dioxigenin as well as haptens and proteins for which labeled antisera or monoclonal antibodies are available.

The particular label used is not critical to the present invention, so long as it does not interfere with the *in situ* hybridization of the probe. In addition the label must be detectible in as low copy number as possible thereby maximizing the sensitivity of the assay and yet be detectible above any background signal. Finally, a label must be chosen that provides a highly localized signal thereby providing a high degree of spatial resolution when physically mapping the probe against the chromosome. In a preferred embodiment, the label is digoxigenin-11-dUTP or biotin-14-dATP, which are then detected using fluorophores.

The labels may be coupled to the probes in a variety of means known to those of skill in the art. In a preferred embodiment the probe will be labeled using nick translation or random primer extension (Rigby, *et al. J. Mol. Biol.*, 113: 237 (1977) or Sambrook, *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1985)).

In situ Hybridization

Standard *in situ* hybridization techniques are used to probe a given sample. Several guides to the techniques are available, *e.g.*, Gall *et al. Meth. Enzymol.*, 21:470-480 (1981) and Angerer *et al.* in *Genetic Engineering: Principles and Methods* Setlow and Hollaender, Eds. Vol 7, pgs 43-65 (plenum Press, New

York 1985). Briefly, cells, either as single cell suspensions or as tissue preparation are deposited on solid supports such as glass slides and fixed by choosing a fixative which provides the best spatial resolution of the cells and the optimal hybridization efficiency.

Generally, *in situ* hybridization comprises the following major steps: (1) fixation of tissue or biological structure to analyzed; (2) prehybridization treatment of the biological structure to increase accessibility of target DNA, and to reduce nonspecific binding; (3) hybridization of the mixture of nucleic acids to the nucleic acid in the biological structure or tissue; (4) posthybridization washes to remove nucleic acid fragments not bound in the hybridization and (5) detection of the hybridized nucleic acid fragments. The reagent used in each of these steps and their conditions for use vary depending on the particular application.

In some applications it is necessary to block the hybridization capacity of repetitive sequences. In this case, human genomic DNA is used as an agent to block such hybridization. The preferred size range is from about 200 bp to about 1000 bases, more preferably between about 400 to about 800 bp for double stranded, nick translated nucleic acids.

Hybridization protocols for the particular applications disclosed here are described in detail below. Suitable protocols are described in Pinkel *et al. Proc. Natl. Acad. Sci. USA*, 85:9138-9142 (1988) and in EPO Pub. No. 430,402.

Typically, it is desirable to use dual color FISH, in which two probes are utilized, each labelled by a different fluorescent dye. A test probe that hybridizes to the region of interest is labelled with one dye, and a control probe that hybridizes to a different region is labelled with a second dye. A

nucleic acid that hybridizes to a stable portion of the chromosome of interest, such as the centromere region, is often most useful as the control probe. In this way, differences between efficiency of hybridization from sample to sample can be accounted for.

As noted above, the methods of the invention are particularly useful in detecting LOH. In this case, LOH regions distal to 16q21are associated with neoplastic growth. As shown below, detection of the presence of a mutant allele can be used to confirm that the deletion leads to LOH. One of skill will recognize a number of methods for detecting a mutant allele. Examples include southern hybridization, PCR, restriction length polymorphisms, or detection of the gene product using, *e.g.*, immunological techniques.

Sample Preparation

The FISH methods for detecting chromosomal amplifications and deletions associated with cancer described herein can be performed on nanogram quantities of the subject nucleic acids. Paraffin embedded tumor sections can be used, as can fresh or frozen material. Because FISH can be applied to the limited material, a number of types of samples can be used. For instance, small biopsy tissue samples from tumors can be used for touch preparations (*see, e.g.*, Kallioniemi, A. *et al.*, *Cytogenet. Cell Genet.* 60: 190-193 (1992)). Small numbers of cells obtained from aspiration biopsy or cells in bodily fluids (*e.g.*, blood, urine, sputum and the like) can also be analyzed.

EXAMPLE 1

CGH studies suggested that an LOH on chromosome 16q is associated with breast cancer, and that this LOH is due to a physical deletion. To map the location of this LOH in primary breast tumors, we performed dual color FISH (Matsumara *et al.*, *Cancer Res.* 52: 3474-3477 (1992)) with probes distributed

along chromosome 16q. A control probe that hybridizes to the centromere region of chromosome 16 was labelled with one color dye, while the test probes were labelled with a second color dye. Cells were scored to determine if the test probe produced fewer signals than the centromere probe in greater than 50% of all cells. Reduced copy number of the test probe target regions relative to the centromere region is strongly correlated with loss of heterozygosity at other loci.

FISH was performed with probes obtained from Los Alamos. Precise mapping and contiguity of the probes was then confirmed prior to their use in analyzing samples.

Methods

Cosmids or yeast artificial chromosomes (YACs) that contain sequences derived from regions distributed along chromosome 16q were labeled with either digoxigenin-11-dUTP or biotin-14-dATP by nick-translation. PCR based screening of a YAC library was performed using primer pairs for exons 5 and 6 of the human E-cadherin gene to produce an E-cadherin specific probe. Biotinylation was carried out using a nick translation kit (Gibco BRL); the reaction was carried out for 90 min at 15°C. For digoxigenin labeling, digoxigenin-11-dUTP (Boehringer Mannheim) was used instead of biotin-14-dATP.

The sample slides were denatured by incubating in denaturing solution (70% formamide, 2X SSC, pH 7.0) at 73°C for 2.5 min. After denaturation, slides were digested with proteinase K (Sigma) at 37°C for 75 min (0.1 μg/ml for lymphocytes, 0.5 μg/ml for cultured cells, and 1.0 μg/ml for clinical samples, in 20mM Tris, 2mM $CaCl_2$, pH7.5).

Lymphocytes were used as normal control. Cell lines were used for deletion mapping of the E-cadherin gene. Slides were prepared as follows: Touch preparations were prepared from fresh primary tumors by

pressing the cut surface of each tumor to a several clean microscope slides. Cells adhering to the slides were air dried and fixed for 5 min each in three changes of freshly prepared Carnoy's solution (3:1 = methanol: acetic acid). Cell suspensions were prepared from fresh tumors by mechanically disaggregating the tumors. The suspended cells were incubated in hypotonic KCl (75mM) for 15 min at 37°C, fixed in Carnoy's solution, and dropped onto slides. Cell lines were cultured in appropriate culture media in humidified chamber under 5% CO_2 at 37°C. Metaphase spreads from breast cancer cell lines were made after treating these cells with colcemid (0.25μg/ml) for 3 hours. After hypotonic KCl treatment for 15 min at 37°C, the cells were fixed in Carnoy's solution, dropped onto slides and air dried. All slides were stored at -70°C until use under nitrogen gas.

Following denaturation of the samples, a probe mixture was applied to a warmed slide according to standard procedures. The probe mixture contained 20 ng of labelled probe, 0.2 ng of chromosome 16-specific a-satellite probe (control probe), and, as blocking DNA, either 10 μg of human placental DNA (Sigma) for cosmid probes or 2μg of human Cot 1 DNA (Gibco BRL) for a YAC probe. The probes and carrier DNA were in 50% formamide, 2X SSC, and 10% dextran sulfate at pH7.0).

The probes were hybridized to the samples for 18-36 hours under a cover slip at 37°C in a moist chamber. After incubation, the slides were washed 3 times in washing buffer (50%) formamide, 2 X SSC, pH7.0), twice in 2X SSC, once in 0.1X SSC, each wash being carried out for 10 min at 45°C. A final wash in 4X SSC was carried out for 5 min at room temperature.

A three-step sandwich procedure was used to immunocytochemically stain the slides after hybridization and washing. Hybridized biotin-labeled probes were detected with Texas Red avidin (Vector

Laboratories), biotin-labeled goat anti-avidin antibody (Vector Laboratories), and finally Texas Red avidin. The digoxigenin-labeled probes were detected with FITC-labeled sheep anti-digoxigenin antibody (Boehringer Mannheim) and finally FITC-labeled rabbit anti-sheep antibody (Vector Laboratories).

The staining protocol was carried out at room temperature. Before staining, the slides were preblocked in 1% bovine serum albumin (BSA) in 4X SSC for 5 min. The first staining step consisted of 2μg/ml of Texas Red avidin in 1% BSA/4X SSC. The slides were then washed sequentially in 4X SSC, 4X SSC/0.1% Triton X-100, 4X SSC, and finally in PN (a mixture of 0.1M NaH_2PO_4 and 0.1M NaH_2PO_4 (pH 8.0), and 0.1% Nonidet P-40) for 10 min each. The slides were then preblocked by incubating in PNM (5% Carnation dry milk/0.02% sodium azide in PN) for 5 min.

The second step consisted of incubating the slides in PN containing 20 μg/ml FITC-labeled sheep anti-digoxigenin antibody and 5 μg/ml biotin-labeled anti-avidin antibody. Three 10 minute washes in PN followed. The slides were then preblocked by incubating in PMN for 5 min.

The third step was done with FITC-labeled rabbit anti-sheep antibody (50:1 dilution) and 2 μg/ml Texas Red avidin in PN. After three PN washes, nuclei were counterstained with 0.2 μM 4', 6-diamidino-2-phenylindole (DAPI) in an antifade solution.

Results

Cells were scored to determine if the test probe produced less signal relative to that from the control centromere region probe in greater than 50% of all cells. Figure 1 shows the results of this relative copy number analysis at 5 sites along chromosome 16q in a primary breast cancer, B208. Cosmid probes C89 and C25, the two probes most proximal to the centromere, gave a signal nearly equal to the control signal in 95%

and 89%, respectively, of the cells examined. In contrast, the test probes C52, C196, and C21 (which lie more distal than the C89 and C25 probes) gave a much lower signal relative to the control probe in nearly all of the cells.

Twenty-three independent primary breast cancers were analyzed in this way and the results are shown in Figure 2. Deletions of all material distal to 16q13 were detected in 10 tumors and deletions of all material distal to 16q21 were detected in an additional three tumors. Thus, all tumors showing physical deletions on 16q appeared to be deleted distal to 16q21. Eight of the 13 tumors scored as deleted on 16q appeared to carry only one copy of chromosome 16q and 5 appeared to have more than one. Thus, all tumors retained at least one apparently intact copy of chromosome 16q.

These results strongly suggest that the deletions on 16q are unmasking recessive mutations in a putative tumor suppressor gene encoded on 16q. Since all the deletions were mapped distal to 16q21, we reasoned that the suppressor gene must be located just distal to 16q21.

EXAMPLE 2

The E-cadherin gene maps to 16q22.1, just distal to the common deletion at 16q21 shown above. E-cadherin (also known as Arc-1, uvomorulin, L-CAM, and cell-CAM-120/80) is a 120 kD cell surface glycoprotein adhesion molecule that connects epithelial cells in a calcium-dependent homophilic fashion (Nagafuchi *et al.*, *Nature* 329: 341-343 (1987); Ozawa *et al.*, *Cell* 63: 1033-1038 (1990)). Several recent findings have suggested that E-cadherin may be a tumor suppressor gene. For example, loss of E-cadherin expression is associated with dedifferentiation and invasiveness of cancer cells (Umbas, *et al.*, *Cancer Research* 52: 5104-

5109 (1992); Giroldi *et al.*, *Cancer and Metastasis Reviews* 12: 29-37 (1993); Dorudi *et al.*, *Am J Pathol* 142: 981-986 (1993); Mayer, *et al.*, *Cancer Res* 53: 1690-1695 (1993); Bringuier *et al.*, *Cancer Research* 53: 3241-3245 (1993); Gamallo *et al.*, *Am J Pathol* 142: 987-993 (1993); Oka *et al.*, *Cancer Research* 53: 1696-1701 (1993)). E-cadherin expression is inversely correlated with the grade of *in vitro* invasiveness in bladder, pancreas, lung, and breast cancer cells lines (Frixen *et al.*, *J. Cell Biology* 113: 173-185 (1991)). Transfection of human breast cancer cell lines with mouse E-cadherin cDNA reduced invasiveness of the cell lines *in vitro* (Frixen *et al.*, *supra*.). A single amino acid substitution in one of the Ca^{2+} binding sites of E- cadherin causes loss of cell-cell adhesion (Ozawa *et al*, *Cell* 63: 1033-1038 (1990)). Also, E-cadherin has regions of homology with *fat*, a tumor suppressor gene in *Drosophila* (Mahoney *et al.*, *Cell* 67: 853-868 (1991)).

We tested the hypothesis that the 16q deletions are indeed unmasking mutations in the E-cadherin gene by detailed analysis of E-cadherin copy number, sequence, and expression in 11 breast cancer cell lines. FISH analysis of relative copy number at E-cadherin in metaphase spreads and interphase nuclei was accomplished using a YAC selected from a genomic library.

Ten established breast cancer cell lines (BT468, BT474, CAMA1, HS578T, MCF-7, MDA134, MDA361, MDA435, SKBR3, and T47D) were obtained from the American Type Culture Collection (ATCC). One cell line, 60OMPE, was provided by Dr. Helene Smith.

Methods

The test probe for FISH analysis of the E-cadherin locus was a YAC clone that contains the human E-cadherin gene. The appropriate YAC (clone 281D8) obtained by polymerase chain reaction (PCR) screening

of the CEPH YAC library. Two primer sets were used to amplify 145 bp within exon 5 of E-cadherin and 121 bp within exon 6 of the gene.

Primers used to amplify various exons within the E-cadherin gene were as follows:

EX1-2.A: 5'-CGGAAGTCAGTTCAGACTCC-3',
EX1-2.B: 5'-CCTCTCTCCAGCTCC-3',
EX3.A: 5'-TTTGAAGATTGCACC-3',
EX3.B: 5'-CCACTGTATTCAGCG-3',
EX4-5.A: 5'-CGGACGATGATGTGAACAC C-3',
EX4-5.B: 5'-TGAAGATCGGAGGATTATCG-3',
EX5.A: 5'-CCAACAAAGACAAAGAAGGC-3',
EX5.B: 5'-TATGTGGCAATGCGTTCTCTATCC-3',
EX6.A: 5'-TCTCTCACGCTGTGTCATCCAACG-3',
EX6.B: 5'-ACAGACCCCTTAAAGACCTCCT GG-3',
EX7-8.A: 5'-CGGACGATGATGTGAACACC-3',
EX7-8.B: 5'-TGAAGATCGGAGGATTATCG-3',
EX9.A: 5'-AGAACGAGGCTAACG-3',
EX9.B: 5'-TCACTGGATTTGTGG-3',
EX10.A: 5'-TTCTACACGTAGCAGTGACG-3',
EX10.B: 5'-GTTCCATAAATGTGTCTGGC-3',
EX11.A: 5'-CGGATTTGGAGAGACACTGC-3',
EX11.B: 5'-TATGATTAGGGCTGTGTACG-3',
EX12.A: 5'-CAGTTGCTACTGGAACAGGG-3',
EX12.B: 5'-TGGGTCGTTGTACTGAATGG-3',
EX13.A: 5'-AGCTCATGGATAACC-3',
EX13.B: 5'-TCCTCCAAGAATCCC-3',
EX14.A: 5'-CTGCTCTTGCTGTTTCTTCG-3',
EX14.B: 5'-TGGTCCTCTTCTCCGCCTCC-3',
EX14-15.A: 5'-CTGCTCTTGCTGTTTCTTCG-3',
EX14-15.B: 5'-TCCAATTTCATCGGG-3',
EX16.A: 5'-GAAAGCGGCTGATACTGACC-3',
EX16.B: 5'-ACGTGATTTCTGCATTTCCC-3'.

Pairs of primers (EX5.A, EX5.B and EX6.A, EX6.B) were used for screening of the YAC library.

E-cadherin expression was assessed by indirect immunofluorescence staining using an antibody

against the extracellular portion of E-cadherin. Cultured breast cancer cells were scraped from the wall of plastic culture flasks, washed 3 times in phosphate buffered saline and adjusted to $1x10^6$ cells in 100 µl of PBS. Ten microliters of mouse anti-E-cadherin monoclonal antibody E9 (*J. Cell. Biochem.* 34:187 (1987)) was added to each cell suspension and incubated for 30 min at 37°C. The cells were then washed 3 times in PBS, incubated in FITC-labeled sheep anti-mouse Ig antibody (Boehringer Mannheim) for 30 min at 37°C, washed 3 times in PBS and resuspended again in 50 µl of PBS. Fluorescence of the cells were observed under a Zeiss fluorescence microscope.

The structural integrity and expression of the non-deleted E-cadherin allele in the breast cancer cell lines was assessed by non-isotopic SSCP analysis (Yap *et al.*, *Nucleic Acids Res* 20: 145 (1992); Ainsworth *et al.*, *Nucleic Acids Res* 19: 405-406 (1991); Ballhausen *et al.*, *Applied and Theoretical Electrophoresis* 3: 129-131 (1993)) and reverse transcriptase mediated PCR (RT-PCR).

The full length sequence of both the human and murine E-cadherin cDNA have recently been reported (Bussemakers *et al.*, *Molecular Biology Reports* 17: 123-128 (1993)). The genomic organization of the mouse gene, including the intron-exon boundaries, has been deduced (Ringwald *et al.*, *Nucleic Acids Research* 19: 6533-6539 (1991)). The intron-exon boundaries of the human E-cadherin gene were deduced by comparison with the mouse homolog, and PCR primers were designed to amplify as much as possible of each of the predicted 16 exons. The PCR primers, designed using the program Primer Detective (Lowe *et al.*, *Nucleic Acids Research* 18, 1757-1761 (1990)), allowed analysis of approximately 80% of the E-cadherin coding sequence.

The SSCP analysis was carried out as follows. Ten µl of PCR product mixed with 2 µl of loading dye

(0.5% bromophenol blue and 0.5% xylene cyanol in formamide) was heat-denatured at 95°C for 5 min and cooled on ice. The samples were then electrophoresed on 0.8 mm thick non-denaturing 6% or 10% polyacrylamide gels (acrylamide: bisacrylamide=49:1 or 19:1) with or without 2.5% or 5% glycerol in 0.5 x TBE buffer at 4-5 V/cm for 3 to 8 hours at room temperature. Gels were stained for 15 min in 0.5X TBE containing 5 μg/ml ethidium bromide. Double stranded and single stranded DNA were visualized on UV transilluminator. Genomic DNA extracted from normal human lymphocytes was used as control template DNA.

Reverse transcriptase-mediated PCR was carried out as follows. Total cellular RNA was extracted from approximately $5x10^6$ cells using Tri Reagent (Molecular Research Center, Inc.), following the manufacturer's protocol. The structural integrity of the isolated RNA was confirmed by electrophoresis of the RNA through a 1.2% formaldehyde gel. Five micrograms of total RNA from cell lines MCF7, 60OMPE, CAMA1 and 578T was converted to first strand cDNA using the Preamp cDNA synthesis kit (BRL). RT-PCR was performed on 1 μl of the synthesized cDNA template in a 10 ml reaction volume containing 1 mM Mg^{++}, 50 mM Tris-Cl, pH 8.3, 0.25 mg/ml BSA, 0.5% Ficoll 400, 1 mM tartrazine dye, 10 pM each of primers 5U and 10L and 0.5U Taq polymerase (Perkin-Elmer). The RT-PCR reactions were performed in a Idaho Technology Air Thermo-Cycler 1605 using 10 ml capillary tubes (Idaho Technology) under the following conditions: 1 minute hard denaturation at 94°C followed by 30 cycles of (0 sec. @ 94°C, 0 sec. @ 58°C, 25 sec. @ 70°C and a 2 minute final extension at 70°C. The PCR products were loaded directly into a 1.2% agarose gel for size fractionation.

Results

All cell lines tested showed at least one E-cadherin locus, as determined by FISH analysis. The copy number of E-cadherin was reduced relative to the control centromere region copy number in four of the eleven cell lines (MDA134, MDA435, SKBR3, and 600MPE). Another line, CAMA1, had the same number of E-cadherin and centromere hybridization signals. This cell line has two 16q isochromosomes plus two additional chromosomes that have chromosome 16 centromeres and lack E-cadherin signals. This cell line was scored as deleted at the E-cadherin locus, using the rationale that one E-cadherin allele was lost from this cell line through deletion, and the other E-cadherin allele was duplicated through formation of an isochromosome 16q, and duplicated again as the cell became tetraploid for chromosome 16q. Significantly, the three primary tumors showing reduced relative copy number distal to 16q21 (Fig. 2) also showed reduced relative copy number at the E-cadherin locus.

Indirect immunofluorescence staining using an antibody directed against the extracellular portion of E-cadherin revealed that all five cell lines having reduced relative copy number at the E-cadherin locus failed to express E-cadherin. Five of the six cell lines in which the relative copy number of E-cadherin was not significantly reduced expressed E-cadherin. These results are summarized in Table 1, and confirm previous reports (Frixen, *et al.*, *supra.*; Sommers, *et al.*, *Cell Growth and Differentiation* 2: 365-372 (1991)).

The failure of the five cell lines having reduced relative E-cadherin gene copy number to express detectable E-cadherin protein on the cell surface suggested that the remaining E-cadherin allele contained inactivating mutations. The structural integrity and expression of the remaining E-cadherin allele were analyzed by non-isotopic SSCP analysis and

reverse transcriptase mediated polymerase chain reaction (RT-PCR).

All five cell lines that have reduced relative copy number at the E-cadherin locus were found to carry DNA mutations in the remaining E-cadherin allele, or did not produce detectable E-cadherin mRNA. MDA134 and SKBR3 were found to have deletions in the E-cadherin gene, as PCR failed to amplify sequences in these exons. Specifically, exon 6 is deleted in MDA134 and exons 3 through 12 are deleted in SKBR3. SSCP analysis of 60OMPE, revealed no mutation or deletions. However, RT-PCR of mRNA from this line, using primers designed to amplify from exon 5 to exon 10, revealed a deletion of approximately 180 bp. The deleted fragment was subcloned and sequenced, revealing a deletion of the 183 bp exon 9.

This result suggested the presence of an intronic mutation that interferes with the normal processing of the E-cadherin message. To characterize the mutation, genomic DNA from normal cells and from 60OMPE were amplified using primers located in exons 8 and 9 (see above) and the resulting approximately 1 kb fragments were cloned and sequenced. This analysis revealed a 22 bp deletion in intron 8 of 60OMPE that eliminates the splice site acceptor sequence.

The cell lines CAMA1 and 57ST were found to be normal in all exons by SSCP. However, RT-PCR analysis showed that these lines did not express detectable E-cadherin mRNA. This suggests that these lines carry mutations that affect either the expression or stability of the E-cadherin message.

Exonic DNA in all of the cell lines that express E- cadherin appeared normal by SSCP analysis, except for one mobility shift detected in DNA fragments amplified from exon 14 in BT468. DNA sequencing analysis of a PCR amplification product from this exon revealed a single base substitution (C→T) at base

position 2526. However, this substitution did not change the amino acid Asn^{751} encoded by this codon (AAC→AAT), so this mutation had no physiological effect. A single polymorphism was detected in exon 13 at base position 2170 (C or T). This substitution does not change the coding amino acid Ala^{692} (GCC→GCT). Two of five E-cadherin positive cell lines (BT474 and MDA361) and 7 of 10 normal cell lines were heterozygous for this polymorphism. However, all six E-cadherin negative cell lines had only one allele at the site of the polymorphism, as expected for cells that had lost one E-cadherin allele through physical deletion.

In summary, these data suggest that E-cadherin is a tumor suppressor gene that is inactivated in breast cancer through deletion of one allele leading to LOH on 16q, and mutation of the other allele. The frequency at which E-cadherin is inactivated in primary breast cancer has not yet been assessed at the genetic level. However, estimates of LOH in mixed breast cancers range from 40 to 50% (Devilee *et al.*, *Oncogene* 6: 1705-1711 (1991); Sato *et al.*, *Cancer Res.* 51: 5794-5799 (1991); Larsson *et al.*, *Genes, Chromosomes and Cancer* 2: 191-197 (1990)). In addition, immunohistochemical analyses of E-cadherin expression showed that expression is reduced in 71% of T3/T4 tumors, 74% of tumors with extensive lymph node metastasis, and in 86% of tumors with distant metastases (Oka *et al.*, *Cancer Res.* 53: 1696-1701 (1993)). Loss of E-cadherin has been reported in advanced cancers of the prostate (Umbas *et al.*, *Cancer Res.* 52: 5104-5109 (1992); Giroldi *et al.*, *Cancer and Metastasis Reviews* 12: 29-37 (1993)), colon (Dorudi *et al.*, *Am J Pathol* 142: 981-986 (1993)), stomach (Mayer *et al.*, *Cancer Res* 53: 1690-1695 (1993)) and bladder (Bringuier *et al.*, *Cancer Res.* 53: 3241-3245 (1993)) and abnormal E-cadherin expression in bladder cancer correlates with shorter survival (Bringuier *et al.*,

supra.). Thus, loss of E-cadherin function leading to reduced cell adhesion seems likely to play an important role in the development of invasive potential in a high frequency of breast cancers and other solid tumors.

The frequency of tumors progressing through loss of E-cadherin function may be even higher than suggested by analyses focused solely on E-cadherin, because dysfunction can also be caused by disruption of the E-cadherin-catenin complex that couples E-cadherin to the cellular cytoskeleton (Ozawa *et al.*, *Proc. Natl. Acad. Sci. USA* 87: 4246-4250 (1990)). This may be caused by loss of function in a-, β- or g-catenin (Shimoyama *et al.*, *Cancer Res.* 52: 5770-5774 (1992)), or by phosphorylation of the E-cadherin-catenin complex by activation of the v-src oncogene (Behrens *et al.*, *J. Cell Biol.* 120: 757-766 (1993)). Of course, other genes on 16q also may play a role in cancer progression since the LOH and FISH studies described here and elsewhere frequently involve regions distal to E-cadherin. The Cell Matrix Adhesion Regulator (CMAR) gene at 16q24 (Koyama *et al.*, *Genomics* 16: 264-265 (1993)) is a candidate second tumor suppressor gene on chromosome 16q. CMAR acts to increase cell adhesion to components of the extracellular matrix (Pullman *et al.*, *Nature* 356, 529-532 (1992)).

All of the references cited above are hereby incorporated by reference. For the purposes of clarity and understanding, the invention has been described in these examples and the above disclosure in some detail. It will be apparent, however, that certain changes and modifications may be practiced within the scope of the appended claims.

Table 1 Expression, gene copy number, and aberrations in the human E-cadherin gene in breast cancer cell lines.

Cell lines	E-cadherin Protein Expression	Predominant population 16 centromere/E-cadherin YAC copies/cell	E-cadherin mRNA Expression	Aberrations in genomic DNA of the E-cadherin gene detected by SSCP and RT-PCR
BT468	+	2/4	+	ND
BT474	+	5/5	+	ND
MCF-7	+	5/6	+	ND
MDA361	+	4/4	+	ND
T47D	+	3/3	+	ND
CAMA1	-	4/4*	-	ND
HS578T	-	2/2	-	ND
MDA134	-	2/1**	-	Deletion (exon 6)
MDA435	-	4/2**	-	ND
SKBR3	-	4/2**	-	Deletion (exon 3-exon 12)
600MPE	-	2/1**	+	Splice acceptor site deletion (intron8)

*Isochromosomes **Deletionon of the E-cadherin gene ND: Not detected

WHAT IS CLAIMED IS:

1. A method of detecting a deletion associated with neoplastic growth, the method comprising:

contacting a chromosome sample from a patient with a composition consisting essentially of one or more labeled nucleic acid probes each of which binds selectively to a polynucleotide sequence on human chromosome 16 distal to 16q21 under conditions in which the probe forms a stable hybridization complex with the sequence; and

detecting the hybridization complex.

2. The method of claim 1, wherein the label is selected from the group consisting of digoxigenin and biotin.

3. The method of claim 1, wherein the step of detecting the hybridization complex is carried out by detecting a fluorescent label.

4. The method of claim 3, wherein the fluorescent label is FITC.

5. The method of claim 1, wherein the probe is selected from the group consisting of polynucleotide sequences from C52, C196 and C21.

6. The method of claim 1, wherein the hybridization complex is detected in a metaphase cell.

7. The method of claim 1, wherein the sample is from a breast tumor.

8. The method of claim 1, further comprising detecting E-cadherin expression in a sample from the patient.

9. The method of claim 1, further comprising contacting the sample with a reference probe which binds selectively to a sequence in the centromere of chromosome 16.

10. A method of detecting a deletion associated with neoplastic growth, the method comprising,

contacting a chromosome sample with a composition consisting essentially of one or more labeled nucleic acid probes each of which binds selectively to a polynucleotide sequence on human chromosome 16 distal to 16q21 under conditions in which the probe forms a stable hybridization complex with the sequence;

detecting the hybridization complex; and

detecting a mutant allele of a gene located on human chromosome 16 distal to 16q21.

11. The method of claim 10, wherein the mutant allele is detected using PCR.

12. The method of claim 10, wherein the mutant allele is detected by detecting expression of the gene.

13. The method of claim 10, wherein the gene is E-cadherin.

14. A composition comprising a nucleic acid probe which binds selectively to a polynucleotide sequence on human chromosome 16 distal to 16q21.

15. The composition of claim 14, wherein the probe is labeled.

16. The composition of claim 14, wherein the label is selected from the group consisting of digoxigenin and biotin.

17. The composition of claim 14, wherein the probe is selected from the group consisting of polynucleotide sequences from C52, C196 and C21.

18. A kit for the detection of deletions associated with neoplastic growth, the kit comprising a compartment which contains a nucleic acid probe which binds selectively to a polynucleotide on human chromosome 16 distal to 16q21.

19. The kit of claim 18, wherein the probe is labeled.

20. The kit of claim 19, wherein label is selected from the group consisting of digoxigenin and biotin.

21. The kit of claim 18, wherein the probe is selected from the group consisting of polynucleotide sequences from C52, C196 and C21.

22. The kit of claim 18, further comprising a reference probe specific to chromosome 16 centromere.

23. The kit of claim 18, further comprising Texas red Avidin and biotin-labeled goat anti-avidin antibodies.

FIG. 1

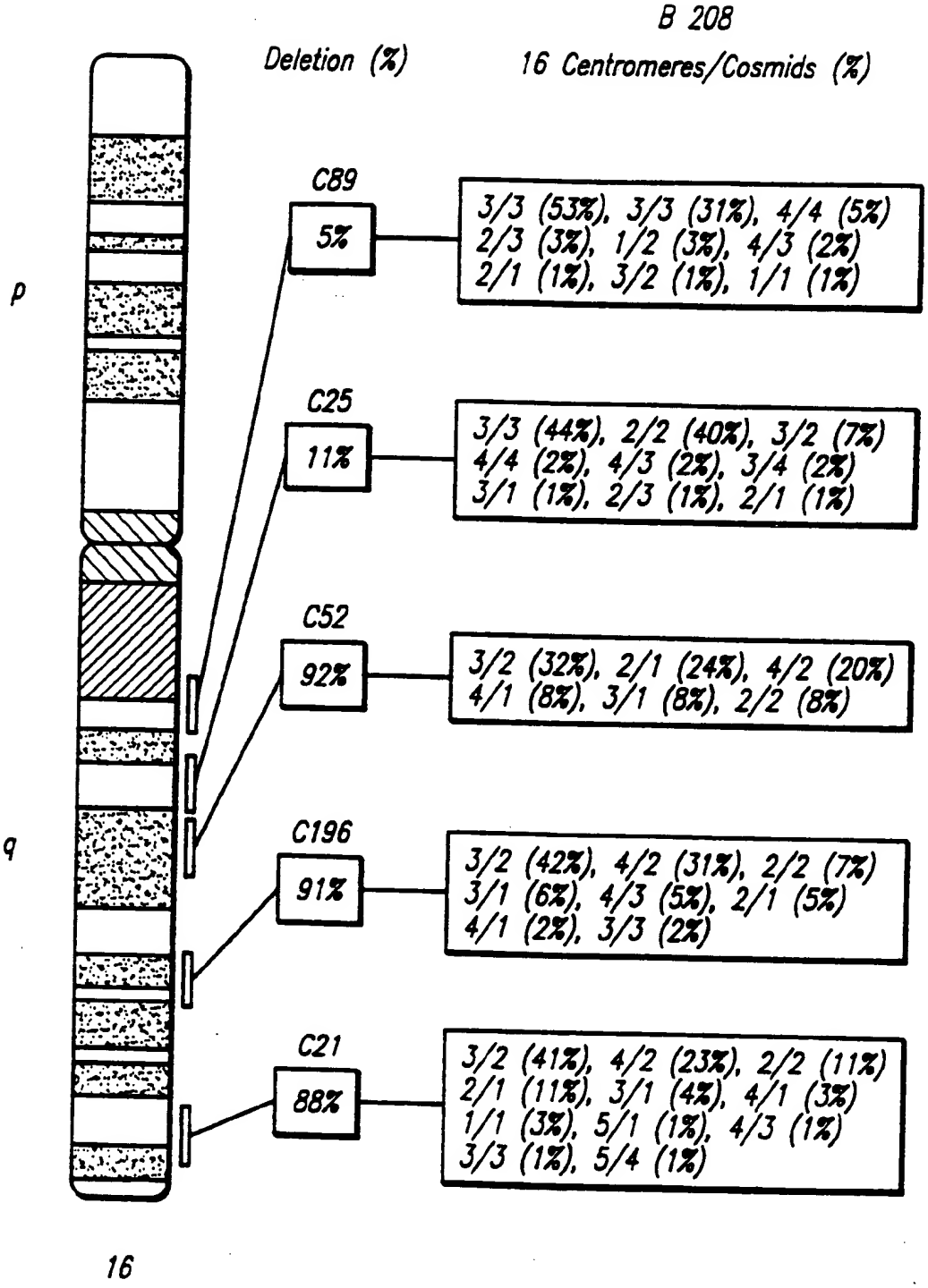
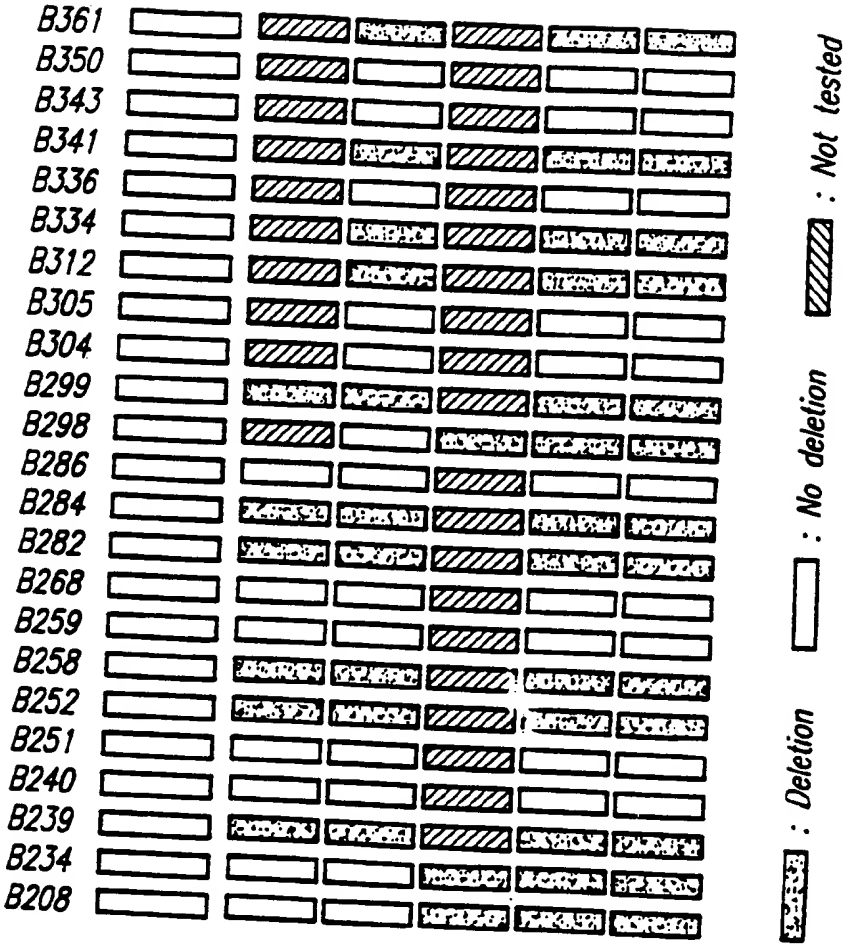


FIG. 2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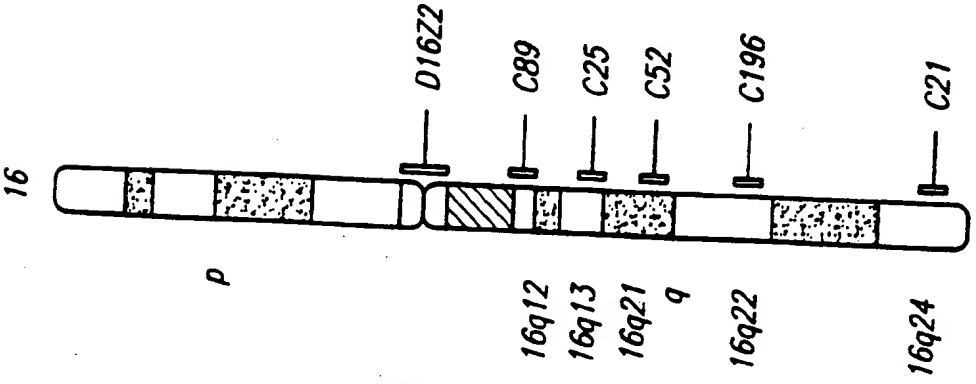
B234
B208
: Deletion
: No deletion
: Not tested


16
D16Z2
C89
C25
C52
C196
C21
p
16q12
16q13
16q21
q
16q22
16q24

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68; C07H 21/02, 21/04

US CL : 435/6; 536/22.1,23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/22.1,23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, APS,CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GENOMICS, VOLUME 13, ISSUED 1992, CALLEN ET AL, "HIGH-RESOLUTION CYTOGENETIC-BASED PHYSICAL MAP OF HUMAN CHROMOSOME 16", SEE PAGES 1178-1185, SEE ENTIRE DOCUMENT.	1-13
Y	CANCER RESEARCH, VOLUME 50, ISSUED 15 NOVEMBER 1990, SATO ET AL, "ALLELOTYPE OF BREAST CANCER: CUMULATIVE ALLELE LOSSES PROMOTE TUMOR PROGRESSION IN PRIMARY BREAST CANCER", PAGES 7184-7189, SEE ENTIRE DOCUMENT.	1-13
Y	CANCER RESEARCH, VOLUME 53, ISSUED 01 APRIL 1993, OKA ET AL, "EXPRESSION OF E-CADHERIN ADHESION MOLECULES IN HUMAN BREAST CANCER TISSUES AND ITS RELATIONSHIP TO METASTASIS", PAGES 1696-1701, SEE ENTIRE DOCUMENT.	1-13

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 NOVEMBER 1994	JAN 19 1995
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Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROC. NATL. ACAD. SCI. USA, VOLUME 87, ISSUED SEPTEMBER 1990, TSUDA ET AL, "ALLELE LOSS ON CHROMOSOME 16 ASSOCIATED WITH PROGRESSION OF HUMAN HEPATOCELLULAR CARCINOMA", PAGES 6791-6794, SEE ENTIRE DOCUMENT.	1-13
Y	CANCER RESEARCH, VOLUME 53, ISSUED AUGUST 1993, LINDBLOM ET AL, "DELETIONS ON CHROMOSOME 16 IN PRIMARY FAMILIAL BREAST CARCINOMAS ARE ASSOCIATED WITH DEVELOPMENT OF DISTANT METASTASES", PAGES 3707-3711, SEE ENTIRE DOCUMENT.	1-13
Y	CANCER RESEARCH, VOLUME 51, ISSUED NOVEMBER 1991, SATO ET AL, "ACCUMULATION OF GENETIC ALTERATIONS AND PROGRESSION OF PRIMARY BREAST CANCER",PAGES 5794-5799, SEE ENTIRE DOCUMENT.	1-20
Y	TECHNIQUE-A JOURNAL OF METHODS IN CELL AND MOLECULAR BIOLOGY, VOLUME 2, NO.3, ISSUED JUNE 1990, POLLARD-KNIGHT, "CURRENT METHODS IN NONRADIOACTIVE NUCLEIC ACID LABELING AND DETECTION", PAGES 113-132, SEE ENTIRE DOCUMENT.	1-23
Y	US, A, 4,996,143 (HELLER ET AL.) 26 FEBRUARY 1991, SEE ENTIRE DOCUMENT.	1-20